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PCT/SE 98/01675

09/508967

REC'D 15 OCT 1998
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Applicant (s)

(21) *Patentansökningsnummer* *9703386-4*
Patent application number

(86) *Ingivningsdatum* *1997-09-19*
Date of filing

Stockholm, 1998-10-09

För Patent- och registreringsverket
For the Patent- and Registration Office

Evy Morin

Avgift
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MALARIA POLYPEPTIDES

Technical field

The present invention relates to carbohydrates capable of acting as receptors for malaria antigens present on the surfaces of malaria infected erythrocytes. In addition, the invention also relates to novel malaria polypeptides capable of acting as ligands in relation to the receptors according to the invention. The invention also encompasses the use thereof as medicaments, pharmaceutical compositions containing the same as well as antibodies directed against said new ligands.

Background and prior art

On a worldwide basis, malaria is one of the most common infectious diseases. Even though it has been largely eliminated from North America and Europe, it remains the most serious infectious disease in tropical and subtropical regions of the world. According to the World Health Organisation, WHO, there are about 100 million new cases each year, and about 300 million people in the developing countries exhibits chronical malaria infections. Malaria is caused by four species of *Plasmodium*, of which *Plasmodium vivax* and *Plasmodium falciparum* are most frequently involved in human infections. The vector responsible for transmitting malaria to humans is the Anopheles mosquito and only malaria infections caused by *Plasmodium falciparum* may be fatal to humans.

After inoculation in the body, the sporozoites of Plasmodium begin to reproduce within liver cells. Multiplication of *Plasmodium* sporozoites occurs by schizogony, in which a single sporozoite can produce as many as 40, 000 merozoites. The invasion of erythrocytes by hepatic merozoites begins the erythrocytic phase of malaria, causing fever and other severe manifestations.

Erythrocytes infected with the malaria parasite *P. falciparum* disappear from the peripheral circulation as they mature from the ring stage to trophozoites. This phenomenon is known as sequestration and results from parasitized erythrocyte adherence to microvascular endothelial cells and erythrocytes in diverse organs. Severe *Plasmodium falciparum*-malaria is characterized by excessive sequestration of infected- and uninfected erythrocytes in the microvasculature of the affected organ.

Thus, *Plasmodium falciparum* is an intracellular protozoan, which during its vertebrate life cycle invades and multiplies in liver and red blood cells. The virulence of the parasite is associated with the capacity of the infected erythrocyte to adhere to endothelial cells and to erythrocytes, so called rosetting. This may cause impaired local oxygen delivery and thereby death of the human host (Miller, L. H., F. Good, and G. Milon. 1994. Malaria pathogenesis. *Science* 264, 1878-1883, Pasloske, B. L. and R.J. Howard. 1994. Malaria, the red cell, and the endothelium. *Ann. Rev. Med.* 45, 283-295, Marsh, K., M. English, J. Crawley, and N. Peshu, 1996. The pathogenesis of severe malaria in African children. *Ann. Trop. Med. & Parasitol.* 90, 395-402). The most malignant form of the infection is cerebral malaria, due to a massive sequestration of infected and uninfected erythrocytes in the brain micro-vasculature.

After being transported from the internal parasite, antigens involved in the binding of cells are thought to be concentrated and subsequently exposed to the exterior of the erythrocyte at minute (≈100 nm in diameter), electron-dense excrescence's called knobs (Atkinson, C. T. and M. Aikawa, 1990. Ultrastructure of malaria-infected erythrocytes. *Blood Cells* 16, 351-368). One such antigen is *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP1), a polypeptide of 200-350 kDa encoded by the *var* family of *P. falciparum* genes (Howard, R. J., J.W. Barnwell, and V. Kao, 1983. Antigenic variation in *Plasmodium knowlesi* malaria: identification of the variant antigen on infected erythrocytes. *Proc.*

Natl. Acad. Sci. U.S.A. 80, 4129-4133, Su, X.-Z, V.M. Heatwole, S.P. Wertheimer, F. Guinet, J.A. Herrfeldt, D.S. Peterson, J.A. Ravetch, and T.E. Wellems. 1995. The large diverse gene family *var* encodes proteins involved in cytoadherence and antigenic variation of *Plasmodium falciparum*-infected erythrocytes. *Cell* 82, 89-100, Baruch, D. I., B.L. Pasloske, H.B. Singh, X. Bi, X.C. Ma, M. Feldman, T.F. Taraschi, and R.J. Howard. 1995. Cloning the *P. falciparum* gene encoding PfEMP1, a malaria variant antigen and adherence receptor on the surface of parasitized human erythrocytes. *Cell* 82, 77-87). The feature of antigenic variation and switching of the surface of the pRBC has been attributed to the *var*-genes. Even though up to 150 such genes are harboured in the genome, only one PfEMP1 is thought to be expressed at any one time. PfEMP1 has features of an adhesive molecule and has been associated with the cytoadherent properties of the infected red cell (Smith, J. D., C.E. Chitnis, A.G. Craig, D.J. Roberts, D.E. Hudson-Taylor, D.S. Peterson, R. Pinches, C.I. Newbold, and L.H. Miller. 1995. Switches in expression of *Plasmodium falciparum var* genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes. *Cell* 82, 101-110, Baruch, D. I., J.A. Gormley, C. Ma, R.J. Howard, and B.L. Pasloske. 1996. *Plasmodium falciparum* erythrocyte membrane protein 1 is a parasitised erythrocyte receptor for adherence to CD36, thrombospondin, and intercellular adhesion molecule 1. *Proc. Natl. Acad. Sci. U.S.A.* 93, 3497-3502). For example, the expression of PfEMP1-encoding *var* genes has been shown to correlate with the capacity of the pRBC for binding to host receptors, including CD36 and ICAM-1 (Baruch, D. I., J.A. Gormley, C. Ma, R.J. Howard, and B.L. Pasloske. 1996. *Plasmodium falciparum* erythrocyte membrane protein 1 is a parasitised erythrocyte receptor for adherence to CD36, thrombospondin, and intercellular adhesion molecule 1. *Proc. Natl. Acad. Sci. U.S.A.* 93, 3497-3502, Gardner, J.P., R.A. Pinches, D.J. Roberts, and C.I. Newbold, (1996). Variant antigens and endothelial receptor adhesion in *Plasmodium falciparum. Proc. Natl. Acad. Sci. U.S.A*, 93, 3503-3508). A role for PfEMP1 in rosetting was recently suggested by Rowe *et al* (Rowe, J.A., J.M. Moulds, C.I. Newbold, and L.H. Miller. 1997. *P.*

falciparum rosetting mediated by a parasite-variant erythrocyte membrane protein and complement-receptor 1. *Nature* 388:292-295). Complement receptor 1 was found to be the host-receptor. However, it is beleived that it cannot be the only one used by rosetting parasites.

In WO 96/33736, one PfEMP1 variant is disclosed by sequence. However, this is only one of several possibly existing variants, all of which are different as to functional sequences as well as adhesive properties.

Accordingly, due to the complex nature and/or mechanism of malarial antigenic variation and the virulence thereof, there is still a great need for methods and compositions which may be useful in the treatment, diagnosis and prevention of malaria infections.

Object of the invention

The present invention fulfills the need described above by providing a carbohydrate, which is useful as a receptor for malaria erythrocyte membrane protein. The invention also provides such a new malaria protein acting as a ligand in relation to the receptors according to the invention.

Breif description of the drawings

Figure 1 discloses the identification of rosetting PfEMP1.

Figure 2 A-D disclose, respectively, maps of cDNA structure, sequencing clones, deduced amino acid sequence and the location of GAG binding motifs in the rosetting PfEMP1 of FCR3S1.2.

Figure 3 discloses the binding of rosetting FCR3S1.2- PfEMP1 to heparine as seen by gel analysis of 10% SDS-PAGE stained with Coomassie.

Figure 4 discloses the disruption of preformed natural rosettes with either one of the fusion proteins DBL-I-GST or ATS-GST.

Figure 5 discloses the effect of glycosaminoglycans on *Plasmodium falciparum* rosetting.

Detailed description of the present invention

More specifically the present invention relates to a carbohydrate, which exhibits at least one negatively charged glycosaminoglycan-like moiety and thereby is capable of essentially specific binding to a malaria erythrocyte membrane protein or an analogue thereof. Accordingly, the carbohydrate according to the invention may be used as a receptor to bind ligands, which are expressed on the surface of cells infected by malaria, such as erythrocytes infected by *Plasmodium falciparum*. In a preferred embodiment, said glycosaminoglycan moiety is sulphated and more specifically, it may be a heparane sulphate moiety.

Indeed, it has been shown in the prior art that *P. falciparum*-rosettes may be disrupted by low concentrations of heparin, an effect that is immediate and reversible (Carlson, J., H.P. Ekre, H. Helmby, J. Gysin, B.M. Greenwood, and M. Wahlgren. 1992. Disruption of *Plasmodium falciparum* erythrocyte rosettes by standard heparin and heparin devoid of anticoagulant activity. *Am. J. Trop. Med. Hyg.* 46, 595-602, Rowe, A., A.R. Berendt, K. Marsh, and C.I. Newbold. 1994. *Plasmodium falciparum*: a family of sulphated glycoconjugates disrupts erythrocyte rosettes. *Exp. Parasitol.* 79, 506-16). However, prior to the present invention, it has not been possible to explain the mechanism or basis for this phenomena and, accordingy, it has not been possible to create any such advantageous and useful receptors as the present receptor carbohydrate until now. Thus, for the first time, the present invention discloses receptors capable of essentially specific binding of such malaria proteins.

The glycosaminoglycans, or GAGs, being the receptor carbohydrate according to the invention, are composed of repeated units of sulfated or acetylated disaccharides and are present in the human body bound to a protein-core in the

form of proteoglycans (PG) (Yanagisha, M. and V. Hascall. 1992. Cell surface heparan sulfate proteoglycans. *J. Biol. Chem.* 267, 9451-9454). Heparan sulfate and chondroitin sulfate are GAGs exposed at all human cell-surfaces, but they are diverse and vary from cell-to-cell and maybe within the same cell due to secondary modifications of the extensive carbohydrate chains (deacetylations, O-sulfations etc.). Heparin is only found in mast-cells and is characterized by a higher degree of sulfation and epimerization than heparan sulfate. However, heparan sulfate and heparin are similar due to heparin-like stretches also found in heparan sulfate (Faham,S., E.R. Hileman, R.J. Fromm, J.R. Lindardt, and C.D. Rees. 1996. Heparin structure and interactions with basic fibroblast growth factor. *Science*, 271, 1116-1120).

In a particular embodiment, the receptor carbohydrate is more specifically capable of essentially specific binding to at least one of the framed segments of the amino acid sequence according to Figure 2D, wherein a novel variant of Plasmodium falciparum erythrocyte membrane protein (PfEMP1) is disclosed. Even though it is more fully discussed elsewhere in this application, especially in connection with the ligand polypeptide below, it should be understood that said framed segments are sequences, which, according to the present invention, have been found to bind specifically and strongly to glycosaminoglycan-like moieties on receptor substances, such as the ones present on most cells or the present polypeptide. Thus, in a specific embodiment, the receptor carbohydrate according to the invention is capable of binding to an amino terminal part, and preferably an essential part, of the framed segments of Figure 2D. Consequently, the protein disclosed in Figure 2D may be regarded as a ligand, whereas the carbohydrate defined as above may be regarded as the receptor therefore. Most preferably, the receptor binds to an essential part of said sequence.

The carbohydrate receptor according to the present invention may be prepared by fractionation from animal cells or by conventional methods, which are well known in the art.

In another aspect, the present invention relates to a carbohydrate as defined above for use as a medicament as well as the use of said receptor as a medicament. Such a medicament is effective in that it is capable of effectively dissolving the rosettes formed by erythrocytes infected by a malaria parasite, as described more detailed in the introductory section of this application. Thereby, a medicament comprising the receptor carbohydrate according to the invention in a suitable form will be effective in dissolving and preventing the occlusion of blood vessels, especially in cerebral malaria. Accordingly, the invention also encompasses the use of said carbohydrate in the manufacture of a medicament against malaria, preferably against *P. falciparum* malaria. Thus, in one embodiment, the invention relates to a pharmaceutical composition comprising said polypeptide in a pharmaceutically or veterinary acceptable carrier. Other additives or excipients which are deemed suitable for the specific application may also be present in such a composition.

One more aspect relating to the above defined carbohydrate, or malaria protein receptor substance, is a method of treating a patient suffering from a malaria infection, preferably a *P. falciparum* infection. Such a method comprises administering to the patient of an effective amount of the pharmaceutical composition described in detail above.

Another aspect of the present invention is a polypeptide, which in practice functions as the ligand in relation to the receptor carbohydrate described above. Said ligand polypeptide originates from a malaria erythrocyte membrane protein and may be denoted a novel PfEMP1-variant.

Thus, by single-cell RT-PCR, the present inventor has identified a novel PfEMP1-variant of a rosetting parasite. Clusters of GAG-binding motifs have been identified in the sequence. In addition, it has been shown that the recombinant form of the novel PfEMP1-variant, or ligand polypeptide according to the invention, adheres to solid-phase heparin, to heparane sulfate on the erythrocyte surface, and disrupts rosettes. Further, naturally formed rosettes also seem to be mediated by binding to the same GAG.

Preferably, the ligand polypeptide according to the invention comprises at least about 300 amino acids of the amino acid sequence according to Figure 2D. Preferably, the ligand comprises an amino terminal sequence of said sequence, or an analogue thereof. More preferably, the ligand according to the invention comprises about 400-500 amino acids, and most preferably about 400 amino acids (DBL-1), such as about 423 amino acids In addition, the ligand polypeptide according to the invention is capable of essentially specific binding to a negatively charged glycosaminoglycan-like moiety. In the preferred embodiment thereof, the ligand polypeptide according to the invention is capable of binding any receptor carbohydrate according to the invention and described above. In a particular embodiment thereof, the ligand polypeptide according to the invention comprises essentially all of the sequence according to Figure 2D.

The weight of the ligand according to the invention may be about 100-300 kDa, preferably about 280 kDa.

Another aspect of the invention is a method of preparing a ligand polypeptide as defined herein, which comprises the steps of

(1) the inserting into an expression vector of a nucleic acid encoding said ligand polypeptide or analogue thereof;

(2) the transfection of a host cell capable of expressing said nucleic acid with said expression vector to express said polypeptide; and

(3) the recovery of the expressed polypeptide. Expression vectors, host cells, process parameters *etc.* are easily chosen by someone skilled in this area. The present invention also relates to a nucleic acid encoding a ligand polypeptide according to the invention, which nucleic acid may be used in the present method. Naturally, the present ligands may also be prepared by synthetic methods, which are well known in the art.

The invention also relates to a nucleic acid encoding the polypeptide according to the invention. Such a nucleic acid may *e.g.* be used in the above method or as a probe *etc.*

Alternatively, the ligand according to the present invention may be produced in the form of a recombinant fusion protein, which comprises said ligand polypeptide, comprising suitable glycosaminglycan-like moieties, fused to another potein or polypeptide. Such a ligand comprising fusion protein may be more advantageous for certain applications than the polypeptide *per se*.

The present invention also relates to a ligand polypeptide according to the invention for use as a medicament as well as the use of the ligand as a a medicament. Such a medicament is effective in that it is capable of effectively dissolving the rosettes formed by erythrocytes infected by a malaria parasite, as described more detailed in the introductory section of this application. Thereby, a medicament comprising the ligand polypeptide according to the invention, *per se* or in the form of a fusion protein, will be effective in dissolving and preventing the occlusion of blood vessels, esecially in cerebral malaria. In addition, as rosettes have been discovered to be associated with other severe complications of malaria (Carlson, J., et al, Lancet 336, 1457-1460 (1990); Treutiger et al, Am. J. Trop. Med. Hyg. 46, 503-510 (1992); and Rowe, A et al, Inf. Immun 63, 2323-2326 (1995)) as well, a medicament comprising a receptor polypeptide according to the invention may also be effective in the treatment of such a condition. Accordingly,

the invention also encompasses the use of said ligand in the manufacture of a medicament against malaria, preferably against *P. falciparum* malaria. The invention also encompasses the use of said ligand in the manufacture of a medicament against other severe complications of malaria. Thus, in one embodiment, the invention relates to a pharmaceutical composition comprising said ligand polypeptide in a pharmaceutically or veterinary acceptable carrier. Other additives or excipients which are deemed suitable for the specific application may also be present in such a composition. Accordingly, another aspect of the invention is a method of treating a patient suffering from a malaria infection by the administration of the present ligand or a fusion protein comprising the ligand. The method comprises administering to said patient of an effective amount of the herein described pharmaceutical composition. The malaria infection most effectively treated by the present composition is a *P. falciparum* infection.

Another highly interesting use of the ligand polypeptide as defined above is the use as a model substance for identifying substances binding to a malaria erythrocyte membrane protein or analogues thereof. Thereby, new receptor substances effective in the treatment and/or prevention of malaria infections may be isolated from suitable environments or bulks comprising the same.

One further aspect of the invention is an antibody, which is specifically immunoreactive with a ligand polypeptide according to the present invention. Such an antibody may be used to formulate another pharmaceutical composition together with suitable pharmaceutically and/or veterinary acceptable carriers, excipients etc. Accordingly, the present invention also relates to such antibody compositions as well as to methods of treating and/or preventing malaria infection in a patient. Such methods comprises administering to said patient of an effective amount of the pharmaceutical composition defined above. Most preferably, the infection to be treated and/or prevented is a *P. falciparum* infection.

As someone skilled in this field easily realises, the receptor carbohydrates, the ligand polypeptide and the antibodies according to the invention are also useful for any diagnostic purposes. Methods, other reagents, amounts etc are easily determined by someone skilled in the area on the basis of the information given in this application.

EXPERIMENTAL

Throughout the present application, the following abbreviations are used: PfEMP 1, Plasmodium falciparum erythrocyte membrane protein 1; DBL, Duffy binding-like; GST, glutathione S-transferase; GAG, glycosaminoglycan; pRBC, parasitised RBC; ICAM-1, intercellular adhesion molecule 1 ; TM, transmembrane.

Materials and Methods

The Parasites

The *P. falciparum* parasites were cultured according to standard methods with 10% AB^+ Rh^+ serum added to the buffered medium (RPMI supplemented with hepes, gentamycin and sodium bicarbonate). The FCR3S1.2 was cloned with micro-manipulation from the previously limiting-dilution cloned parasite FCR3 (Udomsangpetch, R., B. Wåhlin, J. Carlson, K. Berzins, M. Torii, M. Aikawa, P. Perlmann, and M. Wahlgren. 1989. *Plasmodium falciparum*-infected erythrocytes form spontaneous erythrocyte rosettes. *J. Exp. Med.* 169, 1835-1840). Its rosetting rate was routinely >80% (R^+). FCR3S/a was negatively enriched for low rosetting from FCR3 using Ficoll-isopaque (<10%, R^-). It should be noted that FCR3S was previously called Palo Alto (Uganda) in our publications (Carlson, J., H.P. Ekre, H. Helmby, J. Gysin, B.M. Greenwood, and M. Wahlgren. 1992. Disruption of *Plasmodium falciparum* erythrocyte rosettes by standard heparin and heparin devoid of anticoagulant activity. *Am. J. Trop. Med. Hyg.* 46, 595-602, Udomsangpetch, R., B. Wåhlin, J. Carlson, K. Berzins, M. Torii, M. Aikawa, P.

Perlmann, and M. Wahlgren. 1989. *Plasmodium falciparum*-infected erythrocytes form spontaneous erythrocyte rosettes. *J. Exp. Med.* 169, 1835-1840, Scholander, C., C.J. Treutiger, K. Hultenby, and M. Wahlgren. 1996. Novel fibrillar structure confers adhesive property to malaria-infected erythrocytes. *Nature Med.* 2, 204-208, Carlson, J. and M. Wahlgren, 1992. *Plasmodium falciparum* erythrocytes rosetting is mediated by promiscuous lectin-like interactions. *J. Exp. Med.* 176, 1311-1317, Carlson, J., G. Holmquist, D.W. Taylor, P. Perlmann, and M. Wahlgren. 1990. Antibodies to a histidine-rich protein (PfHRP1) disrupt spontaneously formed *Plasmodium falciparum* erythrocyte rosettes. *Proc. Natl. Acad. Sci. U.S.A* 87, 2511-2515, Helmby, H., L. Cavelier, U. Pettersson, and M. Wahlgren. 1993. Rosetting *Plasmodium falciparum*-infected erythrocytes express unique antigens on their surface. *Infect. Immun.* 61, 284-288). Molecular studies of the "Palo Alto" parasites have revealed, however, that they are identical to parasites of the FCR3 lineage (Fandeur, T., S., O. Bonnefoy, Mercereau-Puijalon. 1991. In vivo and in vitro derived Palo Alto lines of *Plasmodium falciparum* are genetically unrelated. *Mol. Biochem. Parasitol.* 47, 167-178).

Optimisation of single P. falciparum RT-PCR

Two degenerate primers (DBL-1.1, 5′-GG(A/T) GC(A/T) TG(TC) GC(A/T) CC(A/T) T(A/T)(T/C) (A/C)G -3′; DBL-1.2, 5′-A(A/G)(A/G)T A(T/C)TG (T/A)GG (A/T)AC (A/G)TA (A/G)TC -3′ which mapped to the conserved region of all PfEMP1 DBL-1 were modified from the sequences of Su *et al.* The amplification parameters were first optimised so that the amplified products were visible with normal ethidium bromide (EB) staining (Cobb, B. D. and J.M. Clarkson, 1994. A simple procedure for optimising the polymerase chain reaction (PCR) using modified Taguchi methods. *Nucl. Acid. Res.* 22, 3801-3805). Briefly, one to five parasites, obtained by limiting dilution, were directly emerged in the RT-PCR buffer (Stratagene) with different concentration of primers, $MgCl_2$, KCl and Tris-Cl. Both DNA and RNA were released from the parasites by heating at 93°C for 3 min. The DNA was degraded by addition of 10 U DNase

(Stratagene). Reverse transcription was carried out immediately after addition of random primers and reverse transcriptase (Perkin-Elmer). The PCR reaction was subsequently performed in the same tube. Through comparison of the amplification efficiency from different reactions, the optimised parameters for single cell RT-PCR were found to be as follows: 100mM Tris-Cl, pH 8.3, 35mM $MgCl_2$, 500mM KCl, and the final concentration of primers was 1μM. In the subsequent experiments, individual trophozoite-infected rosetting erythrocytes were isolated with a 5 μm glass-pipette using an inverted microscope. The selected pRBC was stripped of uninfected RBC and repeatedly grabbed, ejected and turned to conclusively ensure that it had pigment and that the selected cell was a single trophozoite-infected RBC (see Fig. 1A). Fifty cycles of amplification at 93°C for 20 seconds, 55°C for 30 seconds and 72°C for 1 min were needed for product detection. Several controls were included in each experiment; one blank control (without parasite(s)) and one without reverse-transcriptase to rule out the possibility of contamination and amplification due to the presence of genomic DNA.

RT-PCR with total RNA from bulk-cultured FCR3S1.2 or FCR3S/a and Northern-blot analysis

Total RNA purification from both FCR3S1.2 (R^+) and FCR3S/a (R^-) parasite and RT-PCR was performed as described (Sambrook, J., E.F.,Fritsch, and T.,Maniatis. 1989. Molecular Cloning, A laboratory manual, second edition. Cold Spring Harbor Laboratory Press). All the amplified products were cloned with Original TA Cloning Kit (Invitrogen) and sequenced.

Northern blot analysis was carried out using standard methods (Sambrook, J., E.F.,Fritsch, and T.,Maniatis. 1989. Molecular Cloning, A laboratory manual, second edition. Cold Spring Harbor Laboratory Press). Membranes were probed overnight at 60°C using the 434-bp fragment labelled with a-^{32}p-dCTP. Washing was performed under stringent conditions (60°C, 0.1xSSC) and the blots were examined in a Molecular Dynamics phosphoimager.

Cloning and Sequencing of the whole cDNA

A specific upstream primer (L-6, 5′-GAC ATG CAG CAA GGA GCT TGA TAA -3′) in the 434-bp sequence and a downstream primer (L-5, 5'-CCA TCT CTT CAT ATT CAC TTT CTG A -3′) mapping to the conserved sequence of ATS were generated and reverse transcription was carried out as described above. PCR was performed with the Expand™ High Fidelity PCR System (Boehringer Mannheim). A single 4.9-kb fragment was amplified, which was digested into three fragments with Hind III and EcoR V and cloned into the pZErO-1 vector (Zero Background, Invitrogen). The sequencing was performed with LongRanger™ gel (FMC) on an A.L.F. Sequencer (Pharmacia). The 5' region of the FCR3S1.2-*var*1 transcript was cloned by screening a cDNA library (Schlichtherle, unpublished) with the 434-bp fragment as probe and seven overlapping fragments were sequenced. The 3' terminal region was cloned by nested RT-PCR. Reverse transcription was primed with oligo-dT and PCR was performed with a specific 5' primer (P-1, 5′-CTT TCG ACT CTA CCA TCC T -3′) upstream of TM region and a 3' primer (P-4, 5′-TTA GAT ATT CCA TAT ATC TGA TA -3′) mapping to the C-terminal sequence of FCR3 (*var* 2) PfEMP 1. Five overlapping fragments were sequenced. Fourteen overlapping clones were in total sequenced in both directions in order to ensure that the sequence was correct and was transcribed from a single gene.

Sequence analysis

The DNA and amino acid sequence analysis (editing, translation, peptidesort, plot, etc.) was performed with the Genetic Computer Group (GCG) (Devereux, J., P. Haeberli, and O. Smithies. 1984 A comprehensive set of sequence analysis programs for VAX. *Nucl. Acid. Res.* 12, 387-395) program. Alignment of the deduced amino acid sequence with the published PfEMP1 sequences in the GenBank was made to identify the sequence. The identification of potential GAG-binding motifs in FCR3S1.2-*var*1 and in the other published PfEMP1 sequences was done by firstly searching for the presence's of either of the 4 amino-acid

motifs KK, KR, RK, or RR, and secondly manually checking each of the identified sequences for the presence of the motifs XBBXBX or XBBBXXBX where B is a basic amino acid (K,R,H) and X is a hydropathic residue. A certain degree of liberty concerning the location of the basic residues in the motifs is acceptable (Cardin, A. D. and H.J.R. Weintraub, 1989. Molecular modeling of protein-glycosaminoglycan interactions. *Arteriosclerosis* 9, 21-32).

Expression of DBL-1 or ATS using the pGEX-4T-1 vector

Both DBL-1 and ATS fragments were amplified by specific primers (Ex-1.1, 5′-ATC GAA TTC TGC AAA AAA GAT GGA AAA GGA A -3′ and D-1, 5′-GTA TTT TTT TTG TTT GTC AAA TTG -3′ for DBL-1; Ex-2, 5′-ATC GAA TTC TCT GAA AAT TTA TTC CAA A -3′ and P-4 for ATS). The amplified fragments were inserted into the EcoR I cloning site of pGEX-4T-1 downstream of the glutathione S-transferase sequence. The *E.coli* BL21 was used as the expression strain. Expression of both fusion proteins was induced with 0.1mM IPTG at 30°C for 4h and the fusion proteins were purified on glutathione sepharose (Pharmacia) as described in the instructions provided by the manufacturer (GST Gene Fusion System, Pharmacia). The expression constructs were sequenced by cycle sequencing to check that the recombinant plasmids were of the expected sequences in the correct reading frames. Thrombin cleavage of the fusion proteins was performed according to a standard procedure. Western-blot analysis of DBL-1-GST and ATS-GST fusion proteins was with a biotin labelled anti-GST mAb (clone GST-2, IgG2b, Sigma) and ALP-avidin (Sigma) to reveal the pattern of protein expression. Although the induction of expression was at a low temperature and the purification was in the presence of a cocktail of enzyme inhibitors (0.5mM EDTA, 1mM Pefabloc®SC (AEBSF), Boehringer Mannheim), there was still some breakdown of the DBL-1-GST. The fusion proteins, stained by the anti-GST mAb, disappeared with thrombin treatment , leaving a 27 kDa GST protein. This information together with the knowledge that the plasmids we-

re of the expected sequences ensured that the fusion proteins indeed were the correct ones.

Heparin binding and blocking assay

The potential binding of the fusion proteins to heparin was studied by mixing either DBL-1-GST, ATS-GST fusion protein or GST alone (20 µl, 150 µg/ml in PBS) with 20 µl of 50% heparin-sepharose (Pharmacia) and incubating for 5 min at room temperature in the absence of serum; binding to un-coupled sepharose was used as control. The heparin-sepharose and protein mixture was washed 3 times in large volumes of PBST buffer (PBS plus 0.05% Tween-20) before extracting bound proteins in loading-buffer for 10% SDS-PAGE. Inhibition of DBL-1-GST fusion protein binding to heparin with heparin, heparan sulfate or chondroitin sulfate (Lövens Kemiske Fabrik, Denmark, more than 90% pure) was also tested. Twenty µl of 150 µg/ml DBL-1-GST fusion protein was mixed separately for 5 minutes with an equal volume of heparin, heparan sulfate, or chondroitin sulfate, titrated from 10 to 0.5 mg/ml, before the addition of heparin sepharose. The inhibitory activities were checked by SDS-PAGE.

Erythrocyte binding and blocking assay

Ten-well immunofluorescence glass-slides were precoated with 10% poly-L-lysine in PBS for 30 min. Monolayers of RBC were made by addition of 20 µl of 0.5% 3x washed bloodgroup O Rh^+RBC in PBS to each well. Twenty µl DBL-1-GST , ATS-GST or GST alone (80 µg/ml) in PBS was added to the wells for 30 min. The DBL-1-GST fusion protein was in subsequent experiments incubated in the presence of heparin, heparan sulfate or chondroitin sulfate (titrated from 20 to 8 mg/ml) to study the inhibitory activity of each GAG. Slides were washed 3 times with PBS and the fusion protein-binding was detected with the biotin-labelled anti-GST mAb and an ExtraAvidin®FITC conjugate (Sigma). The fluorescence was assessed in a Nikon Optiphot-2 UV microscope, using a x10 ocular and an oil lens with a magnification of x100.

Rosette disruption assay

The assay was performed essentially as described (Carlson, J. and M. Wahlgren, 1992. *Plasmodium falciparum* erythrocytes rosetting is mediated by promiscuous lectin-like interactions. *J. Exp. Med.* 176, 1311-1317). The recombinant fusion proteins (25 ml in PBS of DBL-1-GST or ATS-GST) were added to 25 µl aliquots of a ≈80% rosetting culture of FCR3S1.2 in a micro-titre plate. The mixtures were incubated for 30 min. at 37°C after which the rosetting rate was scored and compared to mock treated controls after staining with acridine orange.

Enzyme treatment of normal RBC

Human bloodgroup O Rh^+ erythrocytes (5% suspension in RPMI) were, previous to C-FDA labelling (Carlson, J., G. Holmquist, D.W. Taylor, P. Perlmann, and M. Wahlgren. 1990. Antibodies to a histidine-rich protein (PfHRP1) disrupt spontaneously formed *Plasmodium falciparum* erythrocyte rosettes. *Proc. Natl. Acad. Sci. U.S.A* 87, 2511-2515), incubated for 60-90 min with either heparinase III (25° C, pH 7.5; Sigma), chondroitinase ABC (37° C, pH 8.0; Sigma) or with C. *perfringens* neuraminidase (37° C, pH 6.0; Sigma). Cells were washed three times after treatment and resuspended in complete malaria medium, with 10% serum. Control erythrocyte suspensions were mock treated, washed and incubated as above.

Detailed description of the drawings

In addition to the description given in this section, the drawings will also be commented under "Results" below.

Figure 1. Identification of rosetting PfEMP1.

Figure 1A rosetting, single *P. falciparum*-infected erythrocyte is seen by light microscopy held by a 5 µm micro-pipette **(A, 1)**. The uninfected erythrocytes are

stripped of the infected cell and careful examination confirms that it indeed is infected by a single parasite (**A, 2-3**).

Figure 1B shows the amplification of a 434-bp band in 4 (from reaction 3, 4, 5 and 7) out of 8 single-infected, rosetting erythrocytes using degenerate primers generated from the primary sequence of the DBL-1 domain of PfEMP1.

Figure 1C shows the amplification pattern with the same primers as in **B** of bulk cultures of rosetting (R^+) FCR3S1.2 cultures and the R^- FCR3s/a parasites. Note that the 434-bp product is only seen with the R^+ parasites.

Figure 1D shows the hybridisation pattern in Northern blotting of the 434-bp sequence to mRNA extracted from the highly rosetting parasite FCR3S1.2 (84% R^+) and the weak hybridisation to the R^- FCR3S/a parasite (9% R^+).

Figure 1E shows the autoradiogarph of a Triton-X 100 insoluble, SDS-soluble extract of FCR3S1.2 infected erythrocytes after radio-iodination labelling. PfEMP1 (arrowed) is labelled on FCR3S1.2 infected erythrocytes and is cleaved by low concentrations of trypsin.

Figure 2. Map of cDNA structure, sequencing clones, deduced amino acid sequence and the location of GAG binding motifs in the rosetting PfEMP1 of FCR3S1.2. **A** shows the location of 434-bp fragment and the three fragments (I, II and III) which were intially cloned for sequencing. Restriction enzyme-digestion sites are indicated by arrows. Additional overlapping clones used for sequencing are shown below. **B** shows the primary structure of the rosetting FCR3S1.2-PfEMP1. It has two Duffy binding-like (DBL) domains (DBL-1 and 4), one cysteine-rich interdomain region (CIDR), one transmembrane (TM) region and one acidic C-terminal segment (ATS). **C** shows the distribution of amino acids in different regions of FCR3S1.2-PfEMP1. **D** shows the complete amino acid sequence of FCR3S1.2 PfEMP1. The location of potential GAG binding motifs are shown in pink. Motifs no.4, 5 and 9,10 (aa 221-232 and 533-549, respectively) are seen as a single stretch as they are located next to each other (see methods

section for description of identification of GAG-binding motifs). These sequence data are available from GenBank under accession number of AF003473.

Figure 3. Rosetting FCR3S1.2-PfEMP1 binds to heparan sulfate. All the gels are 10% SDS-PAGE stained with Coomassie. **A** shows the expressed GST, DBL-1-GST or ATS-GST after purification on glutathione-sepharose and SDS-PAGE. **B** shows the binding capacity of different fusion proteins to heparin-sepharose after SDS-PAGE. **C** shows the inhibition produced by different glycosaminoglycans on the binding of DBL-1-GST to heparin-sepharose followed by SDS-PAGE. **D** & **E** show the binding of DBL-1-GST (**D**) and ATS-GST (**E**) to monolayers of normal RBC as visualised by a mAb to GST labelled with biotin and FITC-avidin.

Figure 4. Disruption of pre-formed, natural rosettes with either DBL-1-GST or ATS-GST.
Results are means and standard errors of three experiments.

Figure 5. Effect of glycosaminoglycans (GAGs) on P. *falciparum* rosetting. **A** shows disruption of rosettes exerted by different GAGs. FCR3S1.2 cultures were incubated with GAGs for 1 h at 37° C and compared to control culture. Results are the means and standard error of three separate experiments. **B** shows the effect of enzyme treatment of uninfected, C-FDA labelled, erythrocytes in a competitive assay of rosette-reformation in the presence of normal erythrocytes and FCR3S1.2 infected pRBC. Results are the means and standard error of three separate experiments; two experiments for neuraminidase.

RESULTS

Identification of a novel rosetting PfEMP1-variant

With the aim of identifying and isolating a PfEMP1-variant of a rosetting parasite, a single-cell RT-PCR assay was developed using micro-manipulation. Since the PfEMP1 messages are mostly expressed at ring stage, it is a difficult task to amplify cDNA from single trophozoites. However, the present inventor was successful in four out of eight pRBC studied and, when amplified, it was always a 434-bp sequence that was found (Fig. 1B). This was confirmed by amplification of *var* transcripts from bulk cultures, where the 434-bp amplificate was again detected unique to the rosetting *P. falciparum* and was not found present in non-rosetting parasites (Fig. 1C). Both the single cell- and the RT-PCR with the total RNA from the bulk cultures were repeated several times to make sure that the results were reproducible. Further, the 434-bp product was also found present in 7 out of 12 ring stage-parasites using single-cell RT-PCR (data not shown). Sequence analysis revealed that the amplified product encoded the semi-conserved sequence of the 5´ located DBL-I domain of the *var* genes. Ten 434-bp sequences obtained from separate amplifications were found to be identical. 9 distinct PCR-amplified *var* transcripts from non-rosetting parasites were also isolated subcloned and sequenced. They were in all instances different from the 434-bp sequence (not shown). Northern-blot analysis with mRNA from the FCR3S1.2 clone and the 434-bp sequence as the labelled probe revealed a transcript of about ≈7.5-kb, the difference with the size of the coding cDNA of 6.7 kb being accounted for by a relatively large untranslated 5' region (Sundström et al, unpublished). The weak hybridisation seen with mRNA from R-parasites is probably due to hybridyzation to a different transcript (Fig. 1D). Thus, according to the present invention, a unique PfEMP1 transcript has been found in rosetting parasites.

cDNA structure of rosetting PfEMP1

The entire coding region of the *var*- transcript containing the 434-bp motif was assembled with the 13 overlapping fragments and the coding sequence was found to be composed of 6684-bp (Fig. 2D). 6684-bp encodes a 2228 aa polypeptide with an estimated molecular weight of 260 kDa. A single trypsin-sensitive polypeptide of a similar size was seen after ^{125}I- lactoperoxidase surface-labelling of the FCR3S1.2 (Fig.1E).

In an in-depth analysis of the expressed FCR3S1.2-*var*1 transcript it was found that the overall structure was similar to the published *var*-sequences. However, the sequence according to the present invention differs substantially from the prior sequences in that it is shorter than most previous sequences, as it contains two DBL-domains (DBL-1 and 4), rather than four, separated by a cysteine-rich interdomain region (CIDR) (Fig. 2B). Both the TM region and the negatively charged, acidic C-terminal segment (ATS) of the FCR3S1.2-*var*1 exhibited a certain degree of homology (≈80%) to previously published sequences. However, neither the potentially variable sequences of 434-bp *var*-gene fragment nor the other regions of the cDNA were contained in other published *var*-sequences, such as the one described in WO 96/33736.

Rosetting PfEMP1 contains clusters of glycosaminoglycan-binding motifs

The FCR3S1.2-*var*1 sequence was examined for the presence of potential GAG-binding motifs. 19 potential GAG-binding sequences were identified and it was found that ≈95% of them (18/19) were located in the N-terminus. 8/19 were situated in DBL-1, 5/19 in the CIDR C-terminally to DBL-1 and 5/19 in DBL-4. Only one potential GAG-binding motif was seen beyond the TM region (Fig. 2D). The N-terminal distribution of these aa clusters is therefore consistent with the accessibility of the FCR3S1.2-*var*1 at the surface of the pRBC.Thus, this may be the molecular background to rosetting and thereby explain why rosettes are sensitive to heparin.

Rosetting PfEMP1 binds to a heparan sulfate-like GAG

To confirm the above findings experimentally, two domains of the FCR3S1.2-*var*1 transcript were subsequently expressed: one which had 8 GAG-binding motifs (DBL-1, 1008-bp corresponding to 336 aa) and one which was the highly charged, acidic C-terminal sequence (ATS) that lacks GAG-binding motifs (1353-bp corresponding to 451 aa). The purified DBL-1-GST efficiently bound to heparin-coupled sepharose already after a few minutes at room temperature while the ATS-GST fusion protein did not (Fig. 3B) and a second DBL-1-GST construct covering a distinct *var* sequence (*var* 2, Su, X.-Z, V.M. Heatwole, S.P. Wertheimer, F. Guinet, J.A. Herrfeldt, D.S. Peterson, J.A. Ravetch, and T.E. Wellems. 1995. The large diverse gene family *var* encodes proteins involved in cytoadherence and antigenic variation of *Plasmodium falciparum*-infected erythrocytes. *Cell* 82, 89-100), which lacks GAG-binding motifs, also failed to bind to the heparin-matrix (not shown). The adhesion could dose-dependently be competed out with heparin or heparan sulfate but not with chondroitin sulfate, another negatively charged erythrocyte surface expressed GAG (Fig. 3C). Taken together, this indicates that the binding of DBL-1 to heparan sulfate is dependent on structure and not merely ionic interactions between the two molecules. Heparan sulfate or a heparan sulfate-like GAG therefore seems to be the binding target for the novel PfEMP1-variant.

Rosetting PfEMP1 binds directly to erythrocytes

To study the potential binding of the recombinant PfEMP1 to erythrocytes we formed monolayers of uninfected erythrocytes on glass-slides. The cells were subsequently incubated with different concentrations of the fusion proteins. While the ATS-GST did not bind to the erythrocytes as detected with an anti-GST mAb, the DBL-1-GST gave a distinct surface staining of all the uninfected erythrocytes (Fig. 3D, E). This was titratable and could be inhibited with small amounts of heparin or heparan sulfate but not with the related GAG, chondroitin sulfate which

suggests that the binding was specific. Heparan sulfate has previously been suggested to be present on human erythrocytes (Trybala, E., B. Svennerholm, T. Bergström, S. Olofsson, S. Jaensson, and J.L. Goodman. 1993. Herpes simplex virus type 1-induced hemagglutination: glycoprotein C mediates virus binding to erythrocyte surface heparan sulfate. J. Virol., 67, 1278-1285, Baggio,B., G. Marzaro, G. Gambaro, F. Marchini, H.E. Williams, and A. Borsatti. 1990. Glycosaminoglycan content, oxalate self-exchange and protein phosphorylation in erythrocytes of patients with idiopathic' calcium oxalate nephrolithiasis. Clinical Science, 79, 113-116) and we therefore conclude that the DBL-1-GST fusion protein binds with heparan sulfate specificity to both to the solid-phase matrix as well as to normal erythrocytes.

Recombinant DBL-1 disrupts rosettes and blocks rosette-reformation

The effect of the DBL-1 fusion protein on pre-formed rosettes was studied in order to confirm the biological role of PfEMP 1 in rosetting. Aliquots of a highly rosetting FCR3S1.2 culture was incubated with decreasing concentrations of DBL-1-GST or ATS-GST. DBL-1 caused a dose-dependent rosette reversion with 40-50% reversion at ≈50 μg/ml while the ATS-GST did not show any effect (Figure 4). The rosettes were not reformed upon prolonged incubation.

Rosetting is dependent on heparan sulfate

To establish the role of heparan sulfate also in rosetting we studied the disruptive activity of different GAGs on FCR3S1.2 rosettes. FCR3S1.2 rosettes were sensitive to both heparin and to heparan sulfate, but neither chondroitin sulfate A, keratan sulfate, nor hyaluronic acid had any effect on the rosettes (Fig. 5A). Chondroitin sulfate B had a slight effect only at high concentrations (Fig. 5A). These findings were confirmed by enzyme treatment of the uninfected erythrocyte: heparinase, but not chondroitinase or neuraminidase, treatment blocked the binding (Fig. 5B).

DISCUSSION

The present invention discloses the identification of a novel PfEMP1-variant as the rosetting ligand and heparan sulfate, or a heparan sulfate like molecule, as the rosetting receptor. A single cell RT-PCR technique was developed in order to investigate the association of expression of the novel PfEMP1-variant with rosetting-binding at the single cell level. The PfEMP 1 messages were found to be present in ring stage parasites, in trophozoites and in schizonts, although it was less abundant in the more mature stage parasites. However, these were studied in great detail as they do form rosettes and rings do not. One PfEMP 1 mRNA species was amplified from single rosetting parasites and the appearance of the same fragment in amplifications with total RNA from R^+ bulk cultures, but not with total RNA from non- or low rosetting parasites, indicated that the message was unique to rosetting parasites. It was evident, however, that the parasites were not homogeneous even in cultures of a high rosetting rate suggesting that the non-rosetting parasites may express different PfEMP 1s. Similarly, although we found strong hybridisation to a ≈7.5 kb message from the rosetting parasites there was still a small hybridisation to a slightly smaller species with RNA from the R^- parasites. This probably reflects the transcription of a different *var* gene, since the FCR1S.2 *var*1 sequence was not found in the amplified products from R- parasites.

Cloning of full length cDNA from eukaryotic cells is always difficult. To be able to achieve this, we first optimised the RT-PCR parameters so that most PfEMP1 transcripts could be amplified. With these conditions and a primer sequence from the unique 434-bp rosetting PfEMP1 cDNA we were able to amplify one large single down-stream fragment of 4.9-kb of cDNA which upon the digestion with EcoR I and Hind III was fragmented into distinct bands indicating that the amplified product was composed of one sequence. The 4.9-kb cDNA species was subsequently confirmed to be a single amplificate by sequencing. Rosetting-PfEMP1

specific sequences of both the variable region, upstream to the trans-membrane domain, and of the 434-bp fragment were used to obtain the sequences of overlapping regions of the 3′ and the 5′ regions of this transcript. Five overlapping fragments were sequenced to ascertain the 3′ overlap and seven to ascertain the 5′ overlap. The correctness was further checked by RT-PCR with specific primers flanking the overlapping regions after the assembly of the entire coding sequence. Each of the 14 PCR- amplified fragments were sequenced in both directions and the overlapping fragments were found to be correct and identical in all regions. It is therefore, highly likely that the assembled transcript is indeed derived from one single gene.

The high sensitivity of rosettes to heparin made us speculate that the parasite uses sulfated carbohydrates as the rosetting receptor. The appearance of potential heparin binding motifs in this novel PfEMP 1 sequence was therefore checked after the assembly of the transcript. Clusters of heparin binding motifs, the consensus sequence of which have previously been proposed by others, were then identified. All the motifs showed an identical or a similar composition as other published heparin or heparan sulfate binding motifs found in both malaria parasites and other organisms (Sinnis, P., P. Clavijo, D. Fenyö, B.T. Chait, C. Cerami, and V. Nussenzweig. 1994. Structural and functional properties of region II-plus of the malaria circumsporozoite protein. *J. Exp. Med.* 180, 297-306, Jackson, L.R., J.S. Busch, and D.A. Cardin. 1991. Glycosaminaoglycans: molecular properties, protein interactions, and role in physiological processes. Physiol. Rev., 71, 481-530). The expressed DBL-1, which had 8 motifs, bound to heparine-sepharose, to the membrane of normal RBC, disrupted naturally formed rosettes and blocked rosette-reformation while the ATS fusion protein did not. The inhibitory activity of heparan sulfate as well as the inability of chondroitin sulfate to inhibit or block the attachment informed us that it was the molecular structure of the GAG that is important for binding. In complementary experiments, the DBL-1 region of a second var-gene transcript (*var*-2) which lacks GAG-binding motifs

was expressed and found not to bind to the heparin-sepharose (Sahlen, not shown) which also support a functional role of the GAG binding sequences in the rosetting DBL-1.

The rosette reversion obtained by the addition of recombinant DBL-1 to naturally formed rosettes indicated that the parasite uses the novel PfEMP1-variant as the main rosetting ligand. The deletion of rosetting by heparinase treatment of normal RBC gave us further confirmation about this specific ligand-receptor interaction indeed suggesting that heparan sulfate, or a heparan sulfate-like molecule, is involved in the binding. Heparinase treatment also disrupted the rosettes of other strains of parasites (TM180, TM284) while the rosettes of the strain R29 were not affected (not shown), findings which are in concordance with those of Rowe *et al* (Rowe, J.A., J.M. Moulds, C.I. Newbold, and L.H. Miller. 1997. *P. falciparum* rosetting mediated by a parasite-variant erythrocyte membrane protein and complement-receptor 1. *Nature* 388:292-295). In separate experiments, heparin-derived mono- and di-saccharides which were of the same net charge but had their sulfate groups at different positions were studied for their rosette-disrupting activities. Those with the sulfate groups in the same position as heparan sulfate inhibited binding while the others did not (Barragan unpublished and see above). Further, while the N-sulfated mono-saccharide glucosamine, a component of heparan sulfate, had a good anti-rosetting activity the identically N-sulfated galactosamine had none (Barragan et al, unpublished). Heparin has also been found to disrupt rosettes from 27 out of 54 fresh *P. falciparum*-isolates (50%) suggesting that heparan sulfate is used by these parasites as rosetting receptors (Carlson, J., H.P. Ekre, H. Helmby, J. Gysin, B.M. Greenwood, and M. Wahlgren. 1992. Disruption of *Plasmodium falciparum* erythrocyte rosettes by standard heparin and heparin devoid of anticoagulant activity. *Am. J. Trop. Med. Hyg.* 46, 595-602). The receptors used by the heparin-resistant fresh isolates maybe other GAGs as we have found that rosettes of most parasites may be disrupted by either one or the other of the human GAGs (Barragan, unpublished). Thus, according to the

present invention, it has been found that FCR3S1.2 uses heparan sulfate, or a heparan sulfate like molecule, on the erythrocyte surface as a rosetting receptor and that the other GAGs may be receptors of other parasites.

Heparan sulfate is a molecule presents on all cells, including erythrocytes (Trybala, E., B. Svennerholm, T. Bergström, S. Olofsson, S. Jaensson, and J.L. Goodman. 1993. Herpes simplex virus type 1-induced hemagglutination: glycoprotein C mediates virus binding to erythrocyte surface heparan sulfate. J. Virol., 67, 1278-1285, Baggio,B., G. Marzaro, G. Gambaro, F. Marchini, H.E. Williams, and A. Borsatti. 1990. Glycosaminoglycan content, oxalate self-exchange and protein phosphorylation in erythrocytes of patients with idiopathic' calcium oxalate nephrolithiasis. Clinical Science, 79, 113-116); 4 x 10^6 molecules have been suggested to be expressed at the surface of normal hepatocytes (Kjellen,L., Å. Oldberg, and M. Höök. 1980. Cell surface heparan sulfate. J Biol. Chem. 21, 10407-10413). The density on the RBC should also be high if this indeed is the receptor used by the parasite for rosetting. The involvement of GAGs as receptor structures for other cell-to-cell interactions of *P. falciparum* has previously been suggested e.g. for endothelial binding , for liver- and erythrocyte invasion (Sinnis, P., P. Clavijo, D. Fenyö, B.T. Chait, C. Cerami, and V. Nussenzweig. 1994. Structural and functional properties of region II-plus of the malaria circumsporozoite protein. *J. Exp. Med.* 180, 297-306, Kulane, A., H-P. Ekre, P. Perlmann, L. Rombo, M. Wahlgren, and B. Wahlin. 1992. Effect of different fractions of heparin on *Plasmodium falciparum* merozoite invasion of red blood cells *in vitro*. *Am. J. Trop.Med. Hyg.*, 46, 589-594, Robert, C., B. Pouvelle, P. Meyer, K. Muanza, A. Scerf, and J. Gysin. 1995. Chondroitin-4-sulfate (proteoglycan) as *Plasmodium falciparum*-infected erythrocyte adherence receptor of brain microvascular endothelial cells. *Res. Immunol.* 146, 383-393, Rogerson, S.J., S.C. Chaiyaroj, K. Ng, J.C. Reeder, and G.V. Brown. 1995. Chondritin sulfate A is a cell surface receptor for *Plasmodium falciparum*-infected erythrocytes. *J. Exp.Med.* 182, 15-20). Taken together this may indicate that the parasite

has adapted a more general strategy for interacting with the host by using the negatively charged proteoglycans as receptors exposed on the exterior of every cell surface.

As a conclusion, the present invention has described the identification, by single-cell RT-PCR and cDNA cloning, of a new adhesive ligand Plasmodium falciparum erythrocyte membrane protein-1-variant , PfEMP1. Rosettes of the novel PfEMP1-variant contains clusters of glycosaminoglycan-binding motifs. A recombinant fusion protein (DBL-1-GST) was found to adhere directly to normal erythrocytes, disrupt naturally formed rosettes, block rosette-reformation and bind to a heparin-sepharose matrix. The adhesive interactions could be inhibited with heparan sulfate or enzymes that remove heparan sulfate from the cell surface while other enzymes or similar glycosaminoglycans of an alike negative charge did not affect the binding. The novel PfEMP1-variant is suggested to be the rosetting ligand and carbohydrates, heparan sulfate, or a heparan sulfate-like molecule, the receptor both for PfEMP1 binding and naturally formed erythrocyte rosettes.

CLAIMS

1. A carbohydrate which exhibits at least one negatively charged glycosaminoglycan-like moiety, whereby it is capable of essentially specific binding to a malaria erythrocyte membrane protein or an analogue thereof.

2. A carbohydrate according to claim 1, wherein said at least one glycosaminoglycan-like moiety is sulphated.

3. A carbohydrate according to claim 1, wherein said at least one glycosaminoglycan like-moiety is a heparan sulphate like moiety.

4. A carbohydrate according to any one of claims 1-3, which more specifically is capable of essentially specific binding to at least one of the framed segments of the amino acid sequence disclosed in Figure 2D or to an analogue thereof.

5. A carbohydrate according to any one of claims 1-4, which is capable of essentially specific binding to an amino terminal part of the amino acid sequence disclosed in Figure 2D or to an analogue thereof.

6. A carbohydrate according to any one of claims 1-5, which is capable of essentially specific binding to essentially all of the framed segments of the amino acid sequence disclosed in Figure 2D or to an analogue thereof.

7. A carbohydrate according to any one of claims 1-6, which is capable of essentially specific binding to the sequence disclosed in Figure 2D or to an analogue thereof.

8. A carbohydrate according to any one of claims 1-7 for use as a medicament.

9. Use of a carbohydrate according to any one of claims 1-7 in the manufacture of a medicament against malaria.

10. A pharmaceutical composition comprising a carbohydrate according to any one of claims 1-7 in a pharmaceutically acceptable carrier.

11. A method of treating a patient suffering from a malaria infection comprising administering to the patient of an effective amount of the pharmaceutical composition according to claim 10.

12. A method according to claim 11, wherein the malaria infection is a *P. falciparum* infection.

13. A polypeptide originating from a malaria erythrocyte membrane protein comprising an amino-terminal part of the sequence according to Figure 2D or an analogue thereof.

14. A polypeptide originating from a malaria erythrocyte membrane protein comprising at least about 300 amino acids of the sequence according to Figure 2D or an analogue thereof.

15. A polypeptide according to claim 13 or 14 comprising about 400-500 amino acids, preferably about 423 amino acids, of the sequence according to Figure 2D or an analogue thereof.

16. A polypeptide according to any one of claims 13-15 capable of essentially specific binding to a negatively charged glycosaminoglycan-like moiety, preferably a sulphated glycosaminoglycan-like moiety.

17. A polypeptide according to any one of claims 13-16 having a weight of about 100-300 kDa, preferably about 280 kDa.

18. A method of preparing a polypeptide according to any one of claims 13-17 or an analogue thereof, which comprises the steps of

(1) the inserting into an expression vector of a nucleic acid encoding said polypeptide or analogue thereof;

(2) the transfection of a host cell capable of expressing said nucleic acid with said expression vector to express said polypeptide; and

(3) the recovery of the expressed polypeptide.

19. A nucleic acid molecule encoding a polypeptide according to any one of claims 13-17.

20. A recombinant fusion protein comprising a polypeptide according to any one of claims 13-17.

21. A polypeptide according to any one of claims 13-17 for use as a medicament.

22. Use of a polypeptide according to any one of claims 13-17 in the manufacture of a medicament for the treatment or prevention of malaria.

23. A pharmaceutical composition comprising a polypeptide according to any one of claims 13-17 in a pharmaceutically acceptable carrier.

24. A method of treating a patient suffering from a malaria infection comprising administering to said patient of an effective amount of the pharmaceutical composition according to claim 23.

25. A method according to claim 24, wherein the malaria infection is a

P. falciparum infection.

26. Use of a polypeptide according to any one of claims 16-18 as a model substance for identifying substances binding to malaria erythrocyte membrane protein or analogues thereof.

27. An antibody which is specifically immunoreactive with a polypeptide according to any one of claims 13-17 or with an analogue thereof.

28. A pharmaceutical composition comprising an antibody according to claim 27 in a pharmaceutically acceptable carrier.

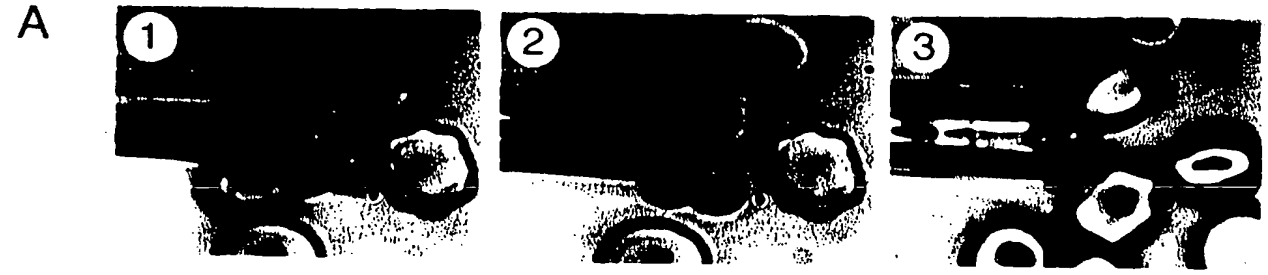
29. A method of treating a patient suffering from a malaria infection comprising administering to said patient of an effective amount of the pharmaceutical composition according to claim 28.

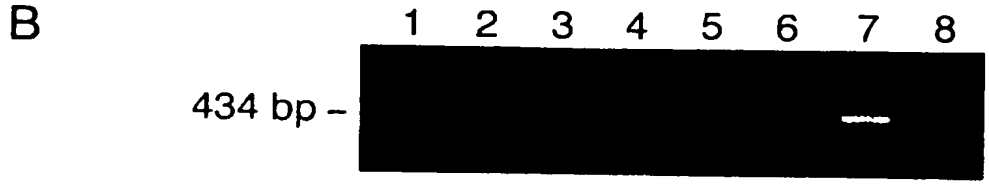
30. A method of preventing malaria in a human or animal object comprising exposure of said human or animal for an effective amount of the pharmaceutical composition according to claim 28.

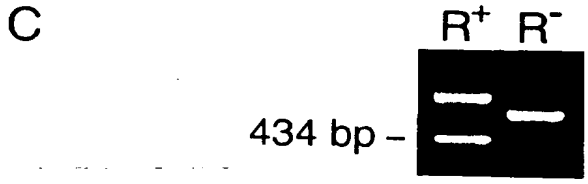
31. A method according to claim 29 or 30, wherein the malaria is *P. falciparum* malaria.

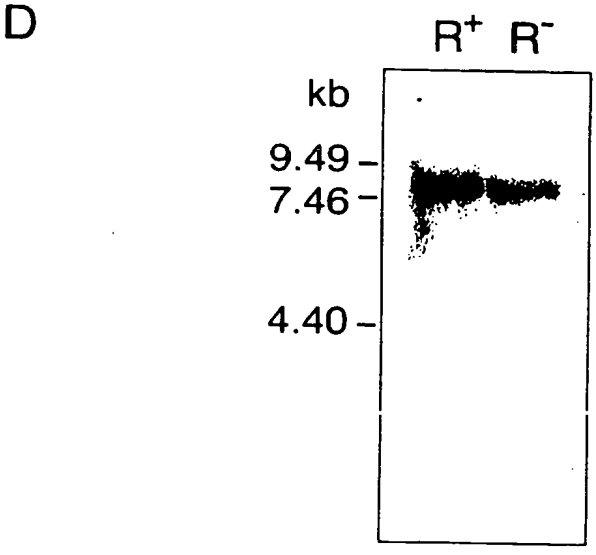
ABSTRACT

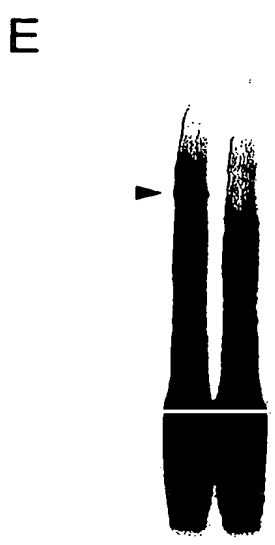
The present invention relates to carbohydrates capable of acting as receptors for malaria antigens present on the surfaces of malaria infected erythrocytes. The receptors according to the invention comprises negatively charged glycosaminoglycan-like moities, preferably sulphated. The invention also relates to novel malaria polypeptides capable of acting as ligands in relation to the receptors according to the invention. The invention also encompasses the use thereof as medicaments, pharmaceutical compositions containing the same as well as antibodies directed against said new ligands.











Rosetting rate (%) 84 9

Trypsin – +

Figure 1

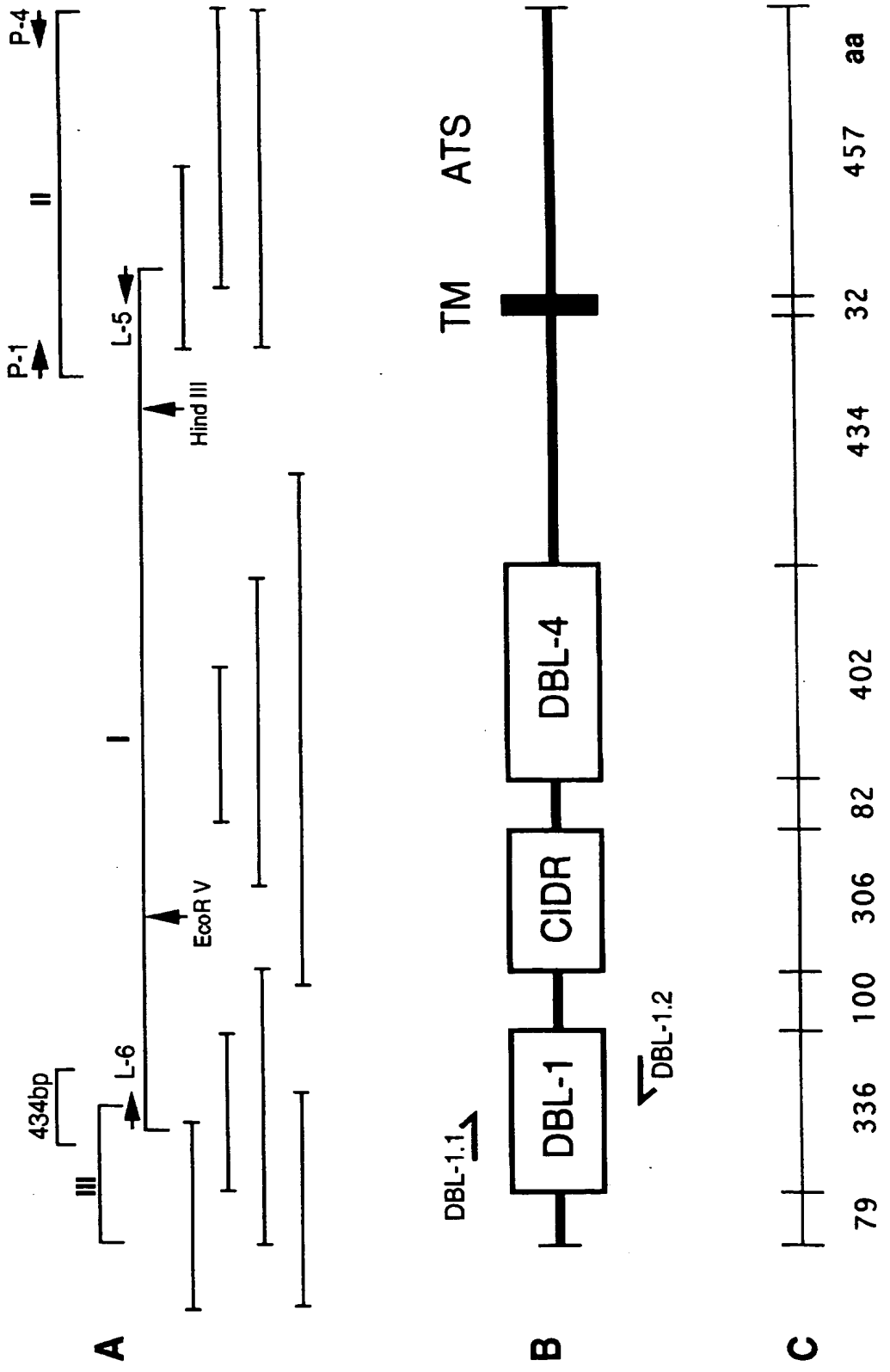

P-4
II
P-1
L-5
Hind III
I
EcoR V
434bp
L-6
III
A
TM
ATS
DBL-4
CIDR
DBL-1
DBL-1.1
DBL-1.2
B
C
aa
457
32
434
402
82
306
100
336
79

Figure 2

```
   1  MATSGGSGGT QDEDAKHVLD EFGQKVHDEV HGEAKNYVSE LKGSLSLAII
  51  LGETAFTVKS MQTESKYTEL IEANSKRNPC KKDGKGNDVD REFSVKEQAY
                                        DBL DOMAIN 1
 101  LNKKMKCSNG MTCAPFRRLH LCNKNFPNMN SNIESSKAKHD LLAEVCMAAL
 151  YEGESIKTHY PKYDSKYPGS DFPMCTMLAR SFADIGDIIR GRDLYLGLKK
 201  KKQNGKETER EKLEQKLKEI FKKIHDNLKD KPAQKRYNGD EDPNFYKLEE
 251  DWWTANRETV WGAMTCSKEL DNSSYFRATC NDTGQGPSQT HNKCRCDKDK
 301  GANAGKPKAG DGDVTIVPTY FDYVPQYLRW FEEWAEDFCR KKKKKLENLE
 351  KQCRGKDKSD EYRYCSRNGY DCEQTISRKG KVRMGKGCTD CFFACHSYEH
 401  WIDNQRKQFD KQKKYTKEIS DGGGRKKRAV GGTTKYEGYE KSFYEKLEHD
 451  GYGTVDAFLG LLNNEKACKD ITDGGKINFK EVNSGGGVVG GQSGGTSGAN
 501  GTNDENKGTF YRSEYCQPCP DCGVQHKGGN QWERKTKVKK MRWSKLYKEI
 551  NGKMVLLLKS LKVVKDMMIL KKNWKEFCLT QNSSDGSVGS VVTTGASGGH
 601  SEKKELYDEW KCYKHNEVQK VNVQGEVEED DDELKGAGGL CILPNPKKNK
 651  EVSEAKSQNN HADIQKTFHD FFYYWVAHML KDSIHWRTKR LKSKCISDGK
 701  MKCRNGCNKK CDCFEKWVKQ KETEWKPIKD HFKTQEGIPE GYYFTTLELI
 751  LKLQFLKEDT EENTENSLDA EEAEELKHLQ KILKLENENN LAVVNAGTEQ
 801  KTLMDKLLNH ELNDATKCKD CPLPEEDKSR GRSADPSPDI FIPRPEEKED
 851  DENEDDDEDE VRDDEETAKE TTEGSATDTT TSLDVCPIVG KVLTKDNESL
 901  QDACSLKYGG NNSRLGWRCV TPSGEPTTSS DKNGAICVPP RRRRLYIKKI
      DBL DOMAIN 4
 951  VDWATKTESP QASGSEASST SGSTTPPDSK EALLKAFVES AAIETFFLMI
1001  RYKEEKKAVA QEGAGHGLPR VEEGSPEYDP EDKLKEGKIP DGFLRQHFYT
1051  LGDYRDILFS GSNDTTSVSK DTPSSSNDNL KNIVLLASGS TEQEREEDIE
1101  YKEIKNFRKC STERSAPNLV SHPQTWWENN GKYIWHGMVC ALTSKDKIAK
1151  GVEKKPQKIE NPENLWDEAN KKPKEPQYQY TNVKLDENSG TSPRTTQTQA
1201  SSDNTPTTLT HFVKRPTYFR WFEEWGESFC PERKKRLKQI KVDCKVENGD
1251  VGRCSGDGEA CDSISTHDYS TVPSFNCPGC GKHCSSYRKW IERKKIEFHK
1301  QSNAYGQQKT DATRNNGNTF DKEFCKTLET VPDAAKFLER LKNGPCETHE
1351  EYGGDDIDFE KDSKTFQHTE YCGPCPKFKT NCQNGNCGVS GLNGNCDGKD
1401  SIDAKEIAKM RSSTTDVVMR VSDNDTNTFE GDDLKDACQH ANIFKGIEED
1451  VWKCGYVCGV DICEQTNINE RTDGKEYIQI RALFKRWVEN FLEDYNKTHD
1501  KISHCIKKGE GSKCINGCEK NSKCLEKWIE KRTAEWENIK KRFNDQYEHK
1551  DQPDYNVKSI LEELIPKIAV VNDQDNVIKL CVFENSKGCT LISNTQNNKE
1601  NDAIDCMLKK LGVKAKNCPG KPSGEKQSDC KEPPPLPDEE DQNPEENTLE
1651  PPKFCPPTTQ PPEEKGGETC GNKEEKKDEK KEESEEPAKE ESGPAAEEPA
1701  PTAESEETET NFPEPPGTGP AAPPSTPAPP TPDTPPPLRP QADEPFDSTI
                                                      TM
1751  LQTTIPFGVA LALGSIAFLF LKKKTKASVG NLFQILQIPK SDYDIPTLKS
1801  SNRYIPYVSD RYKGKTYIYM EGDSDEDKYA FMSDTTDVTS SESEYEELDI
1851  NDIYVPGSPK YKTLIEVVLE PSGNNTTASG KNTPSDTRND IQNDGIPSSK
1901  ITDNEWNQLK KEFISNMLQN QPNDVPNDYT SGNSSTNTNI TTTSRHNVDN
1951  NTNTTMSRDN MEENLLLPSI HDRNLYSGEE YSYNVNMVNS MNDIPINPDH
2001  NVYSGIDLIN DSLSGGKPID IYDEVLKRKE NELFGTENTK RTSTQNVAKI
2051  TNSDPIHNQL ELFHKWLDRH RDMCEKWKNK EDILNKLKEE WNKENIHNSG
2101  KTYNSDNKPS HNHVLNTDVS IQIDMDNPKT KNEITNMDTN QDKSTHDTTL
2151  DDLEKYNDPY YYDFYEDDII YHDVDVEKSS MDDIYVDHNN VTSNIHDVPT
2201  KMHIEHNIVN NKKEIFEEEY FISDIWNT*
```

FIGURE 2 (cont'd)

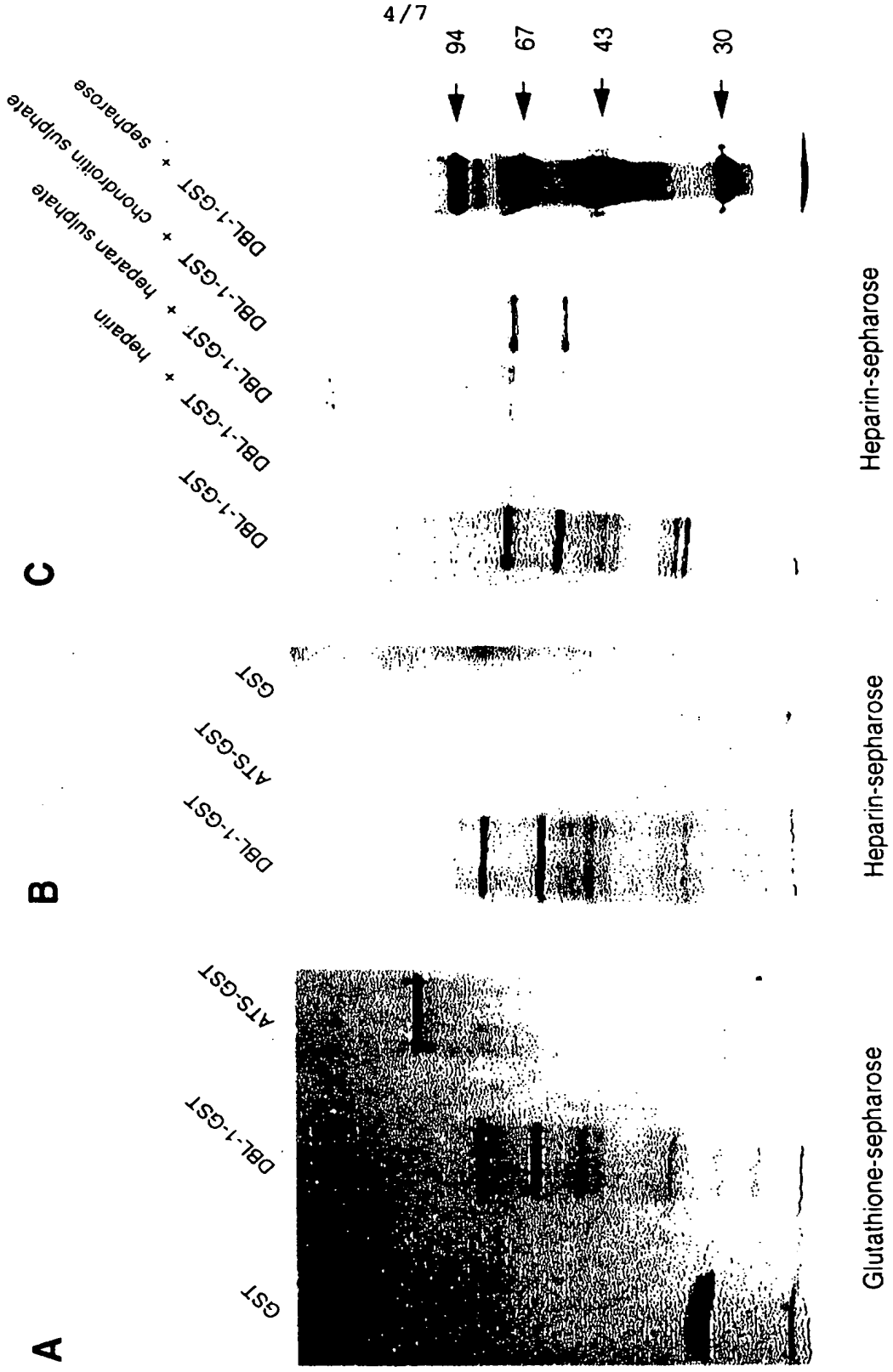


FIGURE 3

Figure 3

D

E

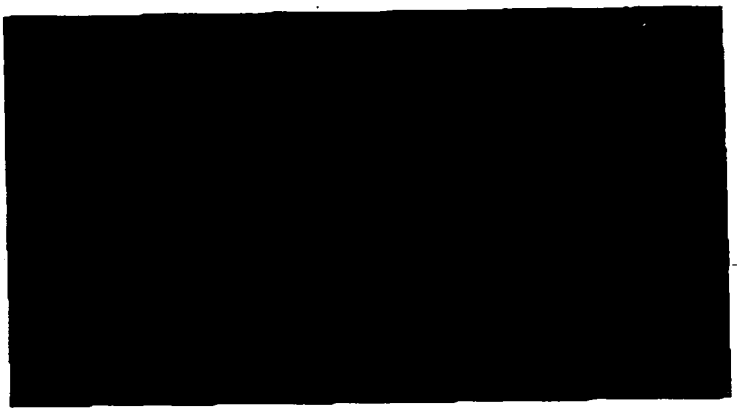


Figure 3 (cont'd)

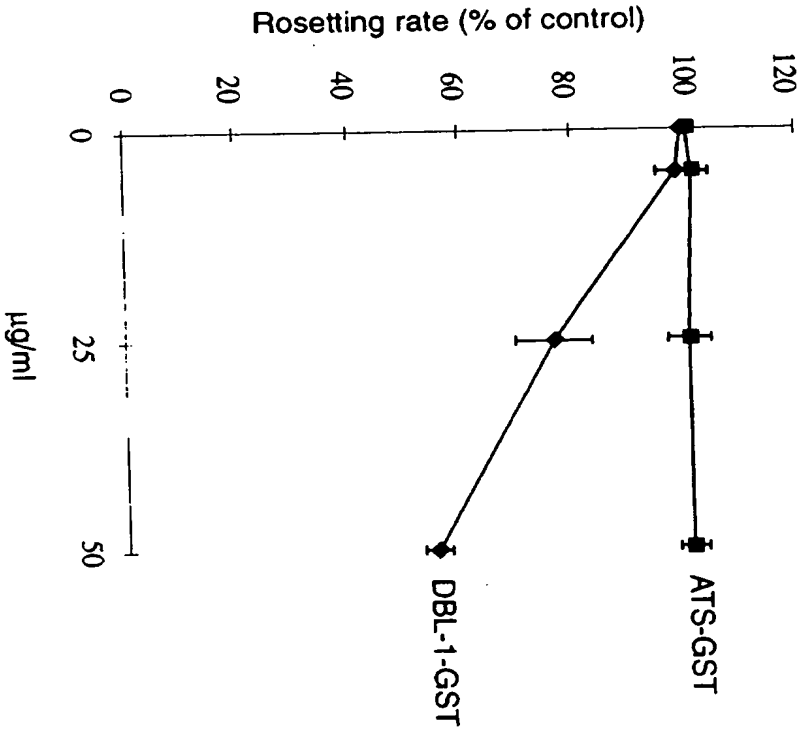

Rosetting rate (% of control)
0
20
40
60
80
100
120
0
25
50
µg/ml
DBL-1-GST
ATS-GST

Figure 4

Figure 5